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<b>(21) International Application Number:</b> PCT/GB97/01414 <b>(22) International Filing Date:</b> 23 May 1997 (23.05.97) <b>(30) Priority Data:</b> 9611981.3 7 June 1996 (07.06.96) GB <b>(71) Applicant (for all designated States except US):</b> ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> DRAKE, Caroline, Rachel [GB/GB]; (GB). BIRD, Colin, Roger [GB/GB]; (GB). SCHUCH, Wolfgang, Walter [DE/GB]; Jealott's Hill Research Station, Bracknell, Berkshire RG42 6ET (GB). <b>(74) Agents:</b> HUSKISSON, Frank, Mackie et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG42 6YA (GB).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> ENHANCEMENT OF GENE EXPRESSION  <b>(57) Abstract</b>  A method for enhancing the expression of a selected gene in an organism while avoiding or reducing co-suppression involves the synthesis of a DNA which is altered in nucleotide sequence and is capable of expression of a protein, ideally identical to that of a protein already expressed by a DNA already present in the organism. This method ensures that sequence similarity between the two genes is reduced enough to eliminate the phenomenon of co-suppression, allowing the over-expression of a specific protein. The method is particularly suitable in plants.  <div data-bbox="1192 1759 1484 1938" style="border: 2px solid black; padding: 10px; text-align: center;"><b>B I</b> <small>PENGAD-Beyens, M. J.</small></div>		

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## ENHANCEMENT OF GENE EXPRESSION

This invention relates to a method and material for enhancing gene expression in organisms, particularly in plants. One particular, but not exclusive, application of the invention is the enhancement of carotenoid biosynthesis in plants such as tomato (*Lycopersicon* spp.)

In order to increase production of a protein by an organism, it is known practice to insert into the genome of the target organism one or more additional copies of the protein-encoding gene by genetic transformation. Such copies would normally be identical to a gene which is already present in the plant or, alternatively, they may be identical copies of a foreign gene. In theory, multiple gene copies should, on expression cause the organism to produce the selected protein in greater than normal amounts, this is referred to as "overexpression". Experiments have shown however, that low expression or no expression of certain genes can result when multiple copies of the gene are present. (Napoli *et al* 1990 and Dorlhac de Borne *et al* 1994). This phenomenon is referred to as co-suppression. It most frequently occurs when recombinant genes are introduced into a plant already containing a gene similar in nucleotide sequence. It has also been observed in endogenous plant genes and transposable elements. The effects of co-suppression are not always immediate and can be influenced by developmental and environmental factors in the primary transformants or in subsequent generations.

The general rule is to transform plants with a DNA sequence the codon usage of which approximates to the codon frequency used by the plant. Experimental analysis has shown that introducing a second copy of a gene identical in sequence to a gene already in the plant genome can result (in some instances) with the expression of the transgene, endogenous gene or both genes being inactivated (co-suppression). The mechanisms of exactly how co-suppression occurs are unclear, however there are several theories incorporating both pre- and post-gene transcriptional blocks.

As a rule the nucleotide sequence of an inserted gene is "optimised" in two respects. The codon usage of the inserted gene is modified to approximate to the preferred codon usage of the species into which the gene is to be inserted. Inserted genes may also be optimised in respect of the nucleotide usage with the aim of

approximating the purine to pyrimidine ratio to that commonly found in the target species. When genes of bacterial origin are transferred to plants, for example, it is well known that the nucleotide usage has to be altered to avoid highly adenylated regions, common in bacterial genes, which may be misread by the eukaryotic expression  
5 machinery as a polyadenylation signal specifying termination of translation, resulting in truncation of the polypeptide. This is all common practice and is entirely logical that an inserted sequence should mimic the codon and nucleotide usage of the target organism for optimum expression.

10 An object of the present invention is to provide means by which co-suppression may be obviated or mitigated.

According to the present invention there is provided a method of enhancing expression of a selected protein by an organism having a gene which produces said protein, comprising inserting into a genome of the said organism a DNA the nucleotide sequence of which is such that the RNA produced on transcription is different from but  
15 the protein produced on translation is the same as that expressed by the gene already present in the genome.

The invention also provides a gene construct comprising in sequence a promoter which is operable in a target organism, a coding region encoding a protein and a termination signal characterised in that the nucleotide sequence of the said  
20 construct is such that the RNA produced on transcription is different from but the protein produced on translation is the same as that expressed by the gene already present in the genome.

The inserted sequence may have a constitutive promoter or a tissue or developmental preferential promoter.

25 It is preferred that the promoter used in the inserted construct be different from that used by the gene already present in the target genome. However, our evidence suggests that it may be sufficient that the region between the transcription and translation initiation codons, sometimes referred to as the "5' intervening region", be different. In other words, the co-suppression phenomenon is probably associated with  
30 the transcription step of expression rather than the translation step: it occurs at the DNA or RNA levels or both.

The invention further provides transgenic plants having enhanced ability to express a selected gene and seed and propagating material derived from the said plant.

This invention is of general applicability to the expression of genes but will be illustrated in one specific embodiment of our invention by a method of enhancing  
5 expression of the phytoene synthase gene which is necessary for the biosynthesis of carotenoids in plants, the said overexpression being achieved by the use of a modified transgene having a different nucleotide sequence from the endogenous sequence.

Preferably said modified phytoene synthase gene has the sequence SEQ-ID-  
10 NO-1.

The invention also provides a modified chloroplast targeting sequence comprising nucleotides 1 to 417 of SEQ-ID-NO-1.

In simple terms, our invention requires that protein expression be enhanced by inserting a gene construct which is altered, with respect to the gene already present in  
15 the genome, by maximising the dissimilarity of nucleotide usage while maintaining identity of the encoded protein. In other words, the concept is to express the same protein from genes which have different nucleotide sequences within their coding region and, preferably the promoter region as well. It is desirable to approximate the nucleotide usage (the purine to pyrimidine ratio) of the inserted gene to that of the  
20 gene already present in the genome. We also believe it to be desirable to avoid the use of codons in the inserted gene which are uncommon in the target organism and to approximate the overall codon usage to the reported codon usage for the target genome.

The degree to which a sequence may be modified depends on the frequency of  
25 degenerate codons. In some instances a high proportion of changes may be made, particularly to the third nucleotide of a triplet, resulting in a low DNA (and consequently RNA) sequence homology between the inserted gene and the gene already present while in other cases, because of the presence of unique codons, the number of changes which are available may be low. The number of changes which are  
30 available can be determined readily by a study of the sequence of the gene which is already present in its degeneracy.

To obtain the gene for insertion in accordance with this invention it may be necessary to synthesise it. The general parameters within which the nucleotide sequence of the synthetic gene compared with the gene already present may be selected are:

- 5 1. Minimise the nucleotide sequence similarity between the synthetic gene and the gene already present in the plant genome;
2. Maintain the identity of the protein encoded by the coding region;
3. Maintain approximately the optimum codon usage indicated for the target genome;
- 10 4. Maintain approximately the same ratio of purine to pyrimidine bases; and
5. Change the promoter or, at least, the 5'-intervening region.

We have worked with the phytoene synthase gene of tomato. The DNA sequence of the endogenous phytoene sequence is known (EMBL Accession Number Y00521): and it was discovered that this gene contained two sequencing errors toward  
15 the 3' end. These errors were corrected in the following way (1) cancel the cytosine at location 1365 and (2) insert a cytosine at 1421. The corrected phytoene synthase sequence (Bartley et al 1992), is given herein as SEQ-ID-NO-2. Beginning with that natural sequence we selected modifications according to the parameters quoted above and synthesised the modified gene which we designated MTOM5 and which has the  
20 sequence SEQ-ID-NO-1. Figure 1 herewith shows an alignment of the natural and synthesised gene with retained nucleotides indicated by dots and alterations by dashes. The modified gene MTOM5 has 63% homology at the DNA level, 100% at the protein level and the proportion of adenine plus thymidine (i.e. the purines) is 54% in the modified gene compared with 58% for the natural sequence.

25 In the sequence listings provided herewith, SEQ ID NO 1 is the DNA sequence of the synthetic (modified TOM5) gene referred to as MTOM5 in Figure 1, SEQ ID NO 2 is the natural genomic phytoene synthase (PsyI) gene referred to as GTOM5 in Figure 1, and SEQ-ID NO 3 is the translation product of both GTOM5 and MTOM5.

In tomato (*Lycopersicon esculentum*), it has been shown that the carotenoid  
30 namely lycopene, is primarily responsible for the red colouration of developing fruit (Bird et al 1991). The production of an enzyme phytoene synthase, referred to herein as PsyI, is an important catalyst in the production of phytoene, a precursor of lycopene.

PsyI catalyses the conversion of geranyl geranyl diphosphate to phytoene, the first dedicated step in carotenoid biosynthesis.

The regulation and expression of the active PsyI gene is necessary for the production of lycopene and consequently the red colouration of fruit during ripening.

5 This can be illustrated by the yellow flesh phenotype of tomato fruits observed in a naturally occurring mutant in which the PsyI gene is inactive. In addition transgenic plants containing an antisense PsyI transgene, which specifically down regulates PsyI expression have also produced the yellow flesh phenotype of the ripe fruit.

When transgenic plants expressing another copy of the PsyI gene (referred to  
10 as TOM5) placed under the control of a constitutive promoter (being the Cauliflower Mosaic Virus 35S promoter) were produced, approximately 30% of the primary transformants produced mature yellow fruit indicative of the phenomenon of co-suppression. Although some of the primary transformants produced an increased carotenoid content, subsequent generations did not exhibit this phenotype thus  
15 providing evidence that co-suppression is not always immediate and can occur in future generations.

The sequence of PsyI is known and hence the amino acid sequence was determined.

With reference to published Genbank genetic sequence data (Ken-nosuke Wada  
20 *et al* 1992.), a synthetic DNA was produced by altering the nucleotide sequence to one which still had a reasonable frequency of codon use in tomato, and which retained the amino acid sequence of PsyI. A simple swap between codons was used in cases where there are only two codon options, however in other cases the codons were changed within the codon usage bias of tomato. Nucleotide sequence analysis indicated that the  
25 synthetic DNA has a nucleotide similarity with PsyI (TOM5 Bartley *et al* 1992) of 63% and amino acid sequence similarity of 100%.

The synthetic gene was then cloned into plant transformation vectors under the control of 35S promoter. These were then transferred into tomato plants by  
*Agrobacterium* transformation, and both the endogenous and the synthetic gene appear  
30 to express the protein. Analysis of the primary transformants illustrates there is no evidence, such as the production of yellow fruit, indicative of co-suppression between the two genes.

The present invention will now be described by way of illustration in the following examples.

### EXAMPLE 1

The coding region of the cDNA which encodes tomato phytoene synthase,  
5 TOM5 (EMBL accession number Y00521) was modified since the original sequence contained two errors towards the 3' end of the sequence. The sequence reported by Bartley et al 1992 (J Biol Chem 267:5036-5039) for TOM5 cDNA homologues therefore differs from TOM5 (EMBL accession number Y00521). For the purpose of the production of the synthetic gene the sequence used is a corrected version of the  
10 TOM5 cDNA which is identical to PsyI (Bartley et al 1992).

#### Design of the sequence.

1. Potential restriction endonuclease cleavage sites were considered given the constraints of the amino acid sequence. Useful sites around the predicted target  
15 sequence cleavage site were introduced to aid subsequent manipulation of the leader.
2. A simple swap between codons was used in cases where there are only two codon options (eg. lysine). In other cases codons were changed within the codon usage bias of tomato as given by Ken-nosuke Wada *et al* (codon usage tabulated from GenBank genetic sequence data, 1992. Nucleic Acids research  
20 20:S2111-2118). A priority was given to reducing homology and avoiding uncommon codons rather than producing a representative spread of codon usage.
3. A BamHI site was introduced at either end of the sequence to facilitate cloning into the initial. At the 5' end 4A were placed upstream of the ATG according  
25 the dicot start site consensus sequence (Cavener and Ray 1991, Eukaryotic start and stop translation sites. NAR 19: 3185-3192).
4. The synthetic gene has been cloned into the vector pGEM4Z such that it can be translated using SP6.
5. Restriction site, stemloop and codon usage analyses were performed, all results  
30 being satisfactory.
6. The modified TOM5 sequence was termed CGS48 or MTOM5.



**Sequence analysis**

CGS48                      AT content = 54%

TOM5                      AT content = 58%

The nucleotide homology between TOM5 and CGS48 is 63%.

- 5    Amino acid sequence homology is 100%.

In summary the sequence TOM5 (Acc. No. Y00521) was extracted from the GenBank database and modified to incorporate the following corrections: deleted C at 1365, inserted C at 1421. CGS48 is based on the CDS of the modified Y00521 and the original sequence, whilst retaining translation product homology and trying to maintain optimal tomato codon usage.

**Assembly of CGS48**

CGS48 was divided into three parts:

CGS48A: BamHI / KpnI

CGS48B: KpnI / SacI

- 15   CGS48C: SacI / BamHI

All three were designed to be cloned on EcoRI / HindIII fragments. The sequences were divided into oligonucleotide fragments following computer analysis to give unique complementarity in the overlapping regions used for the gene assembly.

- 20   The oligonucleotides were synthesised on an Applied Biosystems 380B DNA synthesiser using standard cyanoethyl phosphoramidite chemistry. The oligonucleotides were gel purified and assembled into full length fragments using our own procedures.

The assembled fragments were cloned into pUC18 via their EcoRI/HindIII overhangs.

- 25   Clones were sequenced bi-directionally using "forward" and "reverse" sequencing primers together with the appropriate "build" primers for the top and bottom strands, using the dideoxy-mediated chain termination method for plasmid DNA.

- 30   Inserts from correct CGS48A, B and C clones were isolated by digestion with BamHI / KpnI, KpnI / SacI, SacI / BamHI respectively. The KpnI and SacI ends of the BamHI / KpnI and SacI / BamHI fragments were phosphatased. All three fragments were co-ligated into BamHI cut and phosphatased pGEM4Z. Clones with the correct sized inserts oriented with the 5' end of the insert adjacent to the SmaI site were

identified by PCR amplification of isolated colonies and digestion of purified plasmid DNA with a selection of restriction enzymes.

A CsCl purified plasmid DNA preparation was made from one of these clones. This clone (CGS48) was sequenced bi-directionally using "forward" and "reverse" sequencing primers together with the appropriate "build" primers for the top and bottom strands, using the dideoxy-mediated chain termination method for plasmid DNA.

## EXAMPLE 2

### Construction of the MTOM 5 vector with the CaMV 35S promoter

The fragment MTOM5 (CGS48) DNA described in EXAMPLE 1 was cloned into the vector pJR1Ri (Figure 2) to give the clone pRD13 (Figure 3). The clone CGS48 was digested with SmaI and XbaI and then cloned into pJR1Ri which was cut with SmaI and XbaI to produce the clone pRD13.

## EXAMPLE 3

### Generation and analysis of plants transformed with the vector pRD13

The pRD13 vector was transferred to *Agrobacterium tumefaciens* LBA4404 (a micro-organism widely available to plant biotechnologists) and used to transform tomato plants. Transformation of tomato stem segments followed standard protocols (e.g. Bird et al Plant Molecular Biology 11, 651-662, 1988). Transformed plants were identified by their ability to grow on media containing the antibiotic kanamycin. Forty nine individual plants were regenerated and grown to maturity. None of these plants produced fruit which changed colour to yellow rather than red when ripening. The presence of the pRD13 construct in all of the plants was confirmed by polymerase chain reaction analysis. DNA blot analysis on all plants indicated that the insert copy number was between one and seven. Northern blot analysis on fruit from one plant indicated that the MTOM5 gene was expressed. Six transformed plants were selfed to produce progeny. None of the progeny plants produced fruit which changed colour to yellow rather than red during ripening.

The results are summarised in Table 1 below. The incidence of yellow, or mixed yellow/red (for example, striped) fruits is indicative of suppression of phytoene synthesis. Thus, with the normal GTOM5 construct, 28% of the transgenic plants displayed the co-suppressed phenotype. All the plants carrying the modified MTOM5

construct of this invention had red fruit demonstrating that no suppression of phytoene synthesis had occurred in any of them.

TABLE 1

	Construct	
	35S-GTOM5-nos	35S-MTOM5-nos
Total number of fruiting plants	39	49
Number of plants producing yellow fruit	8	0
Number of plants producing mixed yellow and red fruit or temporal changes	3	0
Number of plants producing red fruit	28	49
% plants showing co-suppression of psyI	28%	0%

5

FIGURE 1

Sequence Alignment of Modified TOM5  
with the synthetic MTOM5

10

TOM5	ATG	TCT	GTT	GCC	TTG	TTA	TGG	GTT	GTT	TCT	30
	...	---	...	...	...	...	...	...	...	---	
MTOM5	ATG	AGC	GTG	GCA	CTT	CTT	TGG	GTG	GTG	AGC	30
	M	S	V	A	L	L	W	V	V	S	
TOM5	CCT	TGT	GAC	GTC	TCA	AAT	GGG	ACA	AGT	TTC	60
	...	...	...	...	---	...	...	...	---	...	
MTOM5	CCA	TGC	GAT	GTG	AGT	AAC	GGC	ACT	TCA	TTT	60
	P	C	D	V	S	N	G	T	S	F	
TOM5	ATG	GAA	TCA	GTC	CGG	GAG	GGA	AAC	CGT	TTT	90
	...	...	---	...	...	...	...	...	...	---	
MTOM5	ATG	GAG	AGT	GTG	AGA	GAA	GGT	AAT	AGA	TTC	90
	M	E	S	V	R	E	G	N	R	F	
TOM5	TTT	GAT	TCA	TCG	AGG	CAT	AGG	AAT	TTG	GTG	120
	...	...	---	...	...	...	...	...	...	---	
MTOM5	TTC	GAC	AGT	TCT	CGT	CAC	CGT	AAC	CTT	GTT	120
	F	D	S	S	R	H	R	N	L	V	
TOM5	TCC	AAT	GAG	AGA	ATC	AAT	AGA	GGT	GGT	GGA	150
	---	...	...	---	...	...	...	...	...	---	
MTOM5	AGT	AAC	GAA	CGT	ATA	AAC	AGG	GGA	GGA	GGT	150
	S	N	E	R	I	N	R	G	G	G	
TOM5	AAG	CAA	ACT	AAT	AAT	GGA	CGG	AAA	TTT	TCT	180
	...	...	...	...	...	...	...	...	...	---	
MTOM5	AAA	CAG	ACA	AAC	AAC	GGT	AGA	AAG	TTC	TCA	180

35

		K	Q	T	N	N	G	R	K	F	S	
	TOM5	GTA	CGG	TCT	GCT	ATT	TTG	GCT	ACT	CCA	TCT	210
5	MTOM5	..-	..-	..-	..-	..-	..-	..-	..-	..-	..-	
		GTT	AGA	TCA	GCA	ATC	CTT	GCA	ACA	CCT	AGC	210
		V	R	S	A	I	L	A	T	P	S	
	TOM5	GGA	GAA	CGG	ACG	ATG	ACA	TCG	GAA	CAG	ATG	240
10	MTOM5	..-	..-	..-	..-	..-	..-	..-	..-	..-	..-	
		GGT	GAG	AGA	ACT	ATG	ACT	AGC	GAG	CAA	ATG	240
		G	E	R	T	M	T	S	E	Q	M	
	TOM5	GTC	TAT	GAT	GTG	GTT	TTG	AGG	CAG	GCA	GCC	270
15	MTOM5	..-	..-	..-	..-	..-	..-	..-	..-	..-	..-	
		GTG	TAC	GAC	GTC	GTA	CTT	CGT	CAA	GCT	GCA	270
		V	Y	D	V	V	L	R	Q	A	A	
	TOM5	TTG	GTG	AAG	AGG	CAA	CTG	AGA	TCT	ACC	AAT	300
20	MTOM5	..-	..-	..-	..-	..-	..-	..-	..-	..-	..-	
		CTA	GTT	AAA	CGT	CAG	TTA	CGT	AGT	ACT	AAC	300
		L	V	K	R	Q	L	R	S	T	N	
	TOM5	GAG	TTA	GAA	GTG	AAG	CCG	GAT	ATA	CCT	ATT	330
25	MTOM5	..-	..-	..-	..-	..-	..-	..-	..-	..-	..-	
		GAA	CTT	GAG	GTT	AAA	CCT	GAC	ATT	CCA	ATA	330
		E	L	E	V	K	P	D	I	P	I	
	TOM5	CCG	GGG	AAT	TTG	GGC	TTG	TTG	AGT	GAA	GCA	360
30	MTOM5	..-	..-	..-	..-	..-	..-	..-	..-	..-	..-	
		CCT	GGA	AAC	CTT	GGA	CTT	CTT	TCT	GAG	GCT	360
		P	G	N	L	G	L	L	S	E	A	
	TOM5	TAT	GAT	AGG	TGT	GGT	GAA	GTA	TGT	GCA	GAG	390
35	MTOM5	..-	..-	..-	..-	..-	..-	..-	..-	..-	..-	
		TAC	GAC	AGA	TGC	GGA	GAG	GTT	TGC	GCA	GAA	390
		Y	D	R	C	G	E	V	C	A	E	
	TOM5	TAT	GCA	AAG	ACG	TTT	AAC	TTA	GGA	ACT	ATG	420
40	MTOM5	..-	..-	..-	..-	..-	..-	..-	..-	..-	..-	
		TAC	GCT	AAA	ACC	TTC	AAT	TTG	GGT	ACC	ATG	420
		Y	A	K	T	F	N	L	G	T	M	
	TOM5	CTA	ATG	ACT	CCC	GAG	AGA	AGA	AGG	GCT	ATC	450
45	MTOM5	..-	..-	..-	..-	..-	..-	..-	..-	..-	..-	
		TTG	ATG	ACA	CCA	GAA	AGG	CGT	CGT	GCA	ATA	450
		L	M	T	P	E	R	R	R	A	I	
	TOM5	TGG	GCA	ATA	TAT	GTA	TGG	TGC	AGA	AGA	ACA	480
50	MTOM5	..-	..-	..-	..-	..-	..-	..-	..-	..-	..-	
		TGG	GCT	ATT	TAC	GTT	TGG	TGT	AGG	CGT	ACT	480
		W	A	I	Y	V	W	C	R	R	T	
	TOM5	GAT	GAA	CTT	GTT	GAT	GGC	CCA	AAC	GCA	TCA	510
55	MTOM5	..-	..-	..-	..-	..-	..-	..-	..-	..-	..-	
		GAC	GAG	TTA	GTG	GAC	GGA	CCT	AAT	GCT	AGT	510
		D	E	L	V	D	G	P	N	A	S	
	TOM5	TAT	ATT	ACC	CCG	GCA	GCC	TTA	GAT	AGG	TGG	540

	MTOM5	..- ..- ..- ..- ..- ..- ..- ..- ..- ... TAC ATA ACA CCC GCT GCT CTT GAC AGA TGG 540 Y I T P A A L D R W
5	TOM5	GAA AAT AGG CTA GAA GAT GTT TTC AAT GGG 570
	MTOM5	..- ..- ..- ..- ..- ..- ..- ..- ..- ... GAG AAC CGT TTG GAG GAC GTG TTT AAC GGC 570 E N R L E D V F N G
10	TOM5	CGG CCA TTT GAC ATG CTC GAT GGT GCT TTG 600
	MTOM5	..- ..- ..- ..- ..- ..- ..- ..- ..- ... AGA CCT TTC GAT ATG TTG GAC GGA GCA CTT 600 R P F D M L D G A L
15	TOM5	TCC GAT ACA GTT TCT AAC TTT CCA GTT GAT 630
	MTOM5	..- ..- ..- ..- ..- ..- ..- ..- ..- ... AGT GAC ACT GTG AGC AAT TTC CCT GTG GAC 630 S D T V S N F P V D
20	TOM5	ATT CAG CCA TTC AGA GAT ATG ATT GAA GGA 660
	MTOM5	..- ..- ..- ..- ..- ..- ..- ..- ..- ... ATC CAA CCT TTT CGG GAC ATG ATC GAG GGC 660 I Q P F R D M I E G
25	TOM5	ATG CGT ATG GAC TTG AGA AAA TCG AGA TAC 690
	MTOM5	..- ..- ..- ..- ..- ..- ..- ..- ..- ... ATG AGA ATG GAT CTT CGT AAG TCT CGT TAT 690 M R M D L R K S R Y
30	TOM5	AAA AAC TTC GAC GAA CTA TAC CTT TAT TGT 720
	MTOM5	..- ..- ..- ..- ..- ..- ..- ..- ..- ... AAG AAT TTT GAT GAG TTG TAT TTG TAC TGC 720 K N F D E L Y L Y C
35	TOM5	TAT TAT GTT GCT GGT ACG GTT GGG TTG ATG 750
	MTOM5	..- ..- ..- ..- ..- ..- ..- ..- ..- ... TAC TAC GTG GCA GGA ACC GTG GGC CTT ATG 750 Y Y V A G T V G L M
40	TOM5	AGT GTT CCA ATT ATG GGT ATC GCC CCT GAA 780
	MTOM5	..- ..- ..- ..- ..- ..- ..- ..- ..- ... TCA GTG CCT ATC ATG GGA ATT GCA CCA GAG 780 S V P I M G I A P E
45	TOM5	TCA AAG GCA ACA ACA GAG AGC GTA TAT AAT 810
	MTOM5	..- ..- ..- ..- ..- ..- ..- ..- ..- ... AGT AAA GCT ACT ACT GAA TCT GTT TAC ACC 810 S K A T T E S V Y N
50	TOM5	GCT GCT TTG GCT CTG GGG ATC GCA AAT CAA 840
	MTOM5	..- ..- ..- ..- ..- ..- ..- ..- ..- ... GCA GCA CTA GCA TTA GGT ATA GCT AAC CAG 840 A A L A L G I A N Q
55	TOM5	TTA ACT AAC ATA CTC AGA GAT GTT GGA GAA 870
	MTOM5	..- ..- ..- ..- ..- ..- ..- ..- ..- ... CTT ACA AAT ATC TTG AGG GAC GTG GGT GAG 870



MTOM5      TTG GTT CCA CCA ACT AAG ACA GCT AGC TTG 1230  
            L    V    P    P    T    K    T    A    S    L

5      TOM5      CAA AGA TAA                      1239  
            .-. -.- .-.  
MTOM5      CAG AGG TGA                      1239  
            Q    R    \*

10              . = Same Base  
                - = Different Base

15              DNA SEQUENCE:              63% HOMOLOGY  
                PROTEIN SEQUENCE:        100% HOMOLOGY

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

(i) APPLICANT: ZENECA LIMITED ., . .

(ii) TITLE OF INVENTION: ENHANCEMENT OF GENE EXPRESSION

10

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:

15

(A) ADDRESSEE: IP DEPT., ZENECA AGROCHEMICALS,

(B) STREET: JEALOTTS HILL RESEARCH STATION,

(C) CITY: BRACKNELL,

(D) STATE: BERKSHIRE

(E) COUNTRY: GB

(F) ZIP: RG42 6ET

20

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

25

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: WO NOT KNOWN

(B) FILING DATE:

(C) CLASSIFICATION:

30

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: HUSKISSON, FRANK M

(C) REFERENCE/DOCKET NUMBER: PPD 50156/WO

35

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 01344 414822

40

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1239 base pairs



(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: SYNTHETIC DNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGAGCGTGG CACTTCTTTG GGTGGTGAGC CCATGCGATG TGAGTAACGG CACTTCATTT  
60

15

ATGGAGAGTG TGAGAGAAGG TAATAGATTC TTCGACAGTT CTCGTCACCG TAACCTTGTT  
120

20

AGTAACGAAC GTATAAACAG GGGAGGAGGT AAACAGACAA ACAACGGTAG AAAGTTCTCA  
180

GTTAGATCAG CAATCCTTGC AACACCTAGC GGTGAGAGAA CTATGACTAG CGAGCAAATG  
240

25

GTGTACGACG TCGTACTTCG TCAAGCTGCA CTAGTTAAAC GTCAGTTACG TAGTACTAAC  
300

GAAGTTGAGG TTAAACCTGA CATTCCAATA CCTGGAAACC TTGGACTTCT TTCTGAGGCT  
360

30

TACGACAGAT GCGGAGAGGT TTGCGCAGAA TACGCTAAAA CCTTCAATTT GGGTACCATG  
420

35

TTGATGACAC CAGAAAGGCG TCGTGCAATA TGGGCTATTT ACGTTTGGTG TAGGCGTACT  
480

GACGAGTTAG TGGACGGACC TAATGCTAGT TACATAACAC CCGCTGCTCT TGACAGATGG  
540

40

GAGAACCGTT TGGAGGACGT GTTTAACGGC AGACCTTTCG ATATGTTGGA CGGAGCACTT  
600

AGTGACACTG TGAGCAATTT CCCTGTGGAC ATCCAACCTT TTCGGGACAT GATCGAGGGC  
660

5 ATGAGAATGG ATCTTCGTAA GTCTCGTTAT AAGAATTTTG ATGAGTTGTA TTTGTACTGC  
720

TACTACGTGG CAGGAACCGT GGGCCTTATG TCAGTGCCTA TCATGGGAAT TGCACCAGAG  
780

10 AGTAAAGCTA CTAAGAATC TGTTTACACC GCAGCACTAG CATTAGGTAT AGCTAACCAG  
840

CTTACAAATA TCTTGAGGGA CGTGGGTGAG GACGCACGTA GGGGTCGTGT GTATCTCCCA  
900

15 CAGGACGAGC TCGCTCAAGC TGGATTGAGT GACGAGGACA TTTTCGCAGG TCGTGTTACA  
960

20 GACAAGTGA GGATTTTCAT GAAAAAGCAG ATTCACCGTG CTCGTAAATT TTTCGACGAA  
1020

GCTGAAAAGG GAGTTACTGA GCTTCTAGT GCATCAAGGT TTCCAGTTG GGCCAGCCTT  
1080

25 GTGCTCTATA GAAAGATTTT GGACGAAATC GAGGCTAACG ATTATAATAA TTTTACTAAA  
1140

CGTGCTTACG TTTCTAAGAG CAAAAAAGCTT ATCGCTCTTC CAATCGCTTA CGCTAAGAGC  
1200

30 TTGGTTCCAC CAACTAAGAC AGCTAGCTTG CAGAGGTGA  
1239

35 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1239 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

40 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

5

(vi) ORIGINAL SOURCE:

(A) ORGANISM: LYOPERSICON ESCULENTUM (TOMATO)

(vii) IMMEDIATE SOURCE:

10

(B) CLONE: GTOM5 - PHYTOENE SYNTHASE GENE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

15

ATGTCTGTTG CCTTGTTATG GGTGTTTCT CTTGTGACG TCTCAAATGG GACAAGTTTC  
60

ATGGAATCAG TCCGGGAGGG AAACCGTTTT TTTGATTCAT CGAGGCATAG GAATTTGGTG  
120

20

TCCAATGAGA GAATCAATAG AGGTGGTGGG AAGCAAATA ATAATGGACG GAAATTTTCT  
180

25

GTACGGTCTG CTATTTTGGC TACTCCATCT GGAGAACGGA CGATGACATC GGAACAGATG  
240

GTCTATGATG TGGTTTGGAG GCAGGCAGCC TTGGTGAAGA GGCAACTGAG ATCTACCAAT  
300

30

GAGTTAGAAG TGAAGCCGGA TATACCTATT CCGGGGAATT TGGGCTTGTT GAGTGAAGCA  
360

TATGATAGGT GTGGTGAAGT ATGTGCAGAG TATGCAAAGA CGTTTAACTT AGGAACTATG  
420

35

CTAATGACTC CCGAGAGAAG AAGGGCTATC TGGGCAATAT ATGTATGGTG CAGAAGAACA  
480

40

GATGAACTTG TTGATGGCCC AAACGCATCA TATATTACCC CGGCAGCCTT AGATAGGTGG  
540

600  
660  
720  
780  
840  
900  
960  
1020  
1080  
1140  
1200  
1239

GAAAAATAGGC TAGAAGATGT TTTCAATGGG CGGCCATTTG ACATGCTCGA TGGTGCTTTG  
TCCGATACAG TTTCTAACTT TCCAGTTGAT ATTCAGCCAT TCAGAGATAT GATTGAAGGA  
ATGCGTATGG ACTTGAGAAA ATCGAGATAC AAAAAGCTTCG ACGAACTATA CCTTTATTGT  
TATTATGTTG CTGGTACGGT TGGGTTGATG AGTGTTCCTT TATGGGTAT CGCCCCTGAA  
TCAAAGGCAA CAACAGAGAG CGTATATAAT GCTGCTTTGG CTCTGGGGAT CGCAAATCAA  
TTAACTAACA TACTCAGAGA TGTTGGAGAA GATGCCAGAA GAGGAAGAGT CTACTTGCCT  
CAAGATGAAT TAGCACAGGC AGGTCTATCC GATGAAGATA TATTTGCTGG AAGGGTGACC  
GATAAATGGA GAATCTTTAT GAAGAAACAA ATACATAGGG CAAGAAAGTT CTTTGATGAG  
GCAGAGAAAG GCGTGACAGA ATTGAGCTCA GCTAGTAGAT TCCCTGTATG GGCATCTTTG  
GTCTTGTAAC GCAAAATACT AGATGAGATT GAAGCCAATG ACTACAACAA CTTACAAAAG  
AGAGCATATG TGAGCAAATC AAAGAAGTTG ATTGCATTAC CTATTGCATA TGCAAAATCT  
CTTGTGCCTC CTACAAAAAC TGCCTCTCTT CAAAGATAA

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 402 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

5 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: LYOPERSICONN ESCULENTUM (TOMATO)

10 (vii) IMMEDIATE SOURCE:

(A) LIBRARY: TRANSLATION PRODUCT OF GTOM5 AND MTOM5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

15

Met Ser Val Ala Leu Leu Trp Val Val Ser Pro Cys Asp Val Ser Asn  
1 5 10 15

20

Gly Thr Ser Phe Met Glu Ser Val Arg Glu Gly Asn Arg Phe Phe Asp  
20 25 30

Ser Ser Arg His Arg Asn Leu Val Ser Asn Glu Arg Ile Asn Arg Gly  
35 40 45

25

Gly Gly Lys Gln Thr Asn Asn Gly Arg Lys Phe Ser Val Arg Ser Ala  
50 55 60

Ile Leu Ala Thr Pro Ser Gly Glu Arg Thr Met Thr Ser Glu Gln Met  
65 70 75 80

30

Val Tyr Asp Val Val Leu Arg Gln Ala Ala Leu Val Lys Arg Gln Leu  
85 90 95

35

Arg Ser Thr Asn Glu Leu Glu Val Lys Pro Asp Ile Pro Ile Pro Gly  
100 105 110

Asn Leu Gly Leu Leu Ser Glu Ala Tyr Asp Arg Cys Gly Glu Val Cys  
115 120 125

40

Ala Glu Tyr Ala Lys Thr Phe Asn Leu Gly Thr Met Leu Met Thr Pro  
130 135 140

	Glu Arg Arg Arg Ala Ile Trp Ala Ile Tyr Val Trp Cys Arg Arg Thr	
	145	150 155 160
5	Asp Glu Leu Val Asp Gly Pro Asn Ala Ser Tyr Ile Thr Pro Ala Ala	
	165	170 175
	Leu Asp Arg Trp Glu Asn Arg Leu Glu Asp Val Phe Asn Gly Arg Pro	
	180	185 190
10	Phe Asp Met Leu Asp Gly Ala Leu Ser Asp Thr Val Ser Asn Phe Pro	
	195	200 205
	Val Asp Ile Gln Pro Phe Arg Asp Met Ile Glu Gly Met Arg Met Asp	
	210	215 220
15	Leu Arg Lys Ser Arg Tyr Lys Asn Phe Asp Glu Leu Tyr Leu Tyr Cys	
	225	230 235 240
	Tyr Tyr Val Ala Gly Thr Val Gly Leu Met Ser Val Pro Ile Met Gly	
20	245	250 255
	Ile Ala Pro Glu Ser Lys Ala Thr Thr Glu Ser Val Tyr Asn Ala Ala	
	260	265 270
25	Leu Ala Leu Gly Ile Ala Asn Gln Leu Thr Asn Ile Leu Arg Asp Val	
	275	280 285
	Gly Glu Asp Ala Arg Arg Gly Arg Val Tyr Leu Pro Gln Asp Glu Leu	
	290	295 300
30	Ala Gln Ala Gly Leu Ser Asp Glu Asp Ile Phe Ala Gly Arg Val Thr	
	305	310 315 320
	Ile His Arg Ala Arg Lys Phe Phe Asp Glu Ala Glu Lys Gly Val Thr	
35	325	330 335
	Glu Leu Ser Ser Ala Ser Arg Phe Pro Val Trp Ala Ser Leu Val Leu	
	340	345 350
40	Tyr Arg Lys Ile Leu Asp Glu Ile Glu Ala Asn Asp Tyr Asn Asn Phe	
	355	360 365

Thr Lys Arg Ala Tyr Val Ser Lys Ser Lys Lys Leu Ile Ala Leu Pro  
370 375 380

5 Ile Ala Tyr Ala Lys Ser Leu Val Pro Pro Thr Lys Thr Ala Ser Leu  
385 390 395 400

Gln Arg

## CLAIMS

1. A method of enhancing expression of a selected protein by an organism having a gene which produces said protein, comprising inserting into the genome of  
5 the said organism a DNA the nucleotide sequence of which is such that the RNA produced on transcription is different from but the protein produced on translation is the same as that expressed by the gene already present in the genome.
- 10 2. A method as claimed in claim 1, in which the organism is a plant.
3. A method as claimed in claim 2, in which the plant is a tomato plant.
4. A method as claimed in any preceding claim, in which the selected gene is the  
15 gene encoding phytoene synthase.
5. A method as claimed in claim 4, in which the coding region of the said inserted gene has the sequence SEQ-ID-NO-1.
- 20 6. A gene construct comprising in sequence a promoter which is operable in a target organism, a coding region encoding a protein and a termination signal characterised in that the nucleotide sequence of the said construct is such that the RNA produced on transcription is different from but the protein produced on translation is the same as that expressed by the gene already present in the  
25 genome.
7. A method of enhancing expression of carotenoids in a plant comprising overexpression in the plant a gene specifying an enzyme necessary to the biosynthesis of carotenoids, the said overexpression being achieved by the use  
30 of a modified transgene having a different nucleotide sequence from the endogenous sequence.

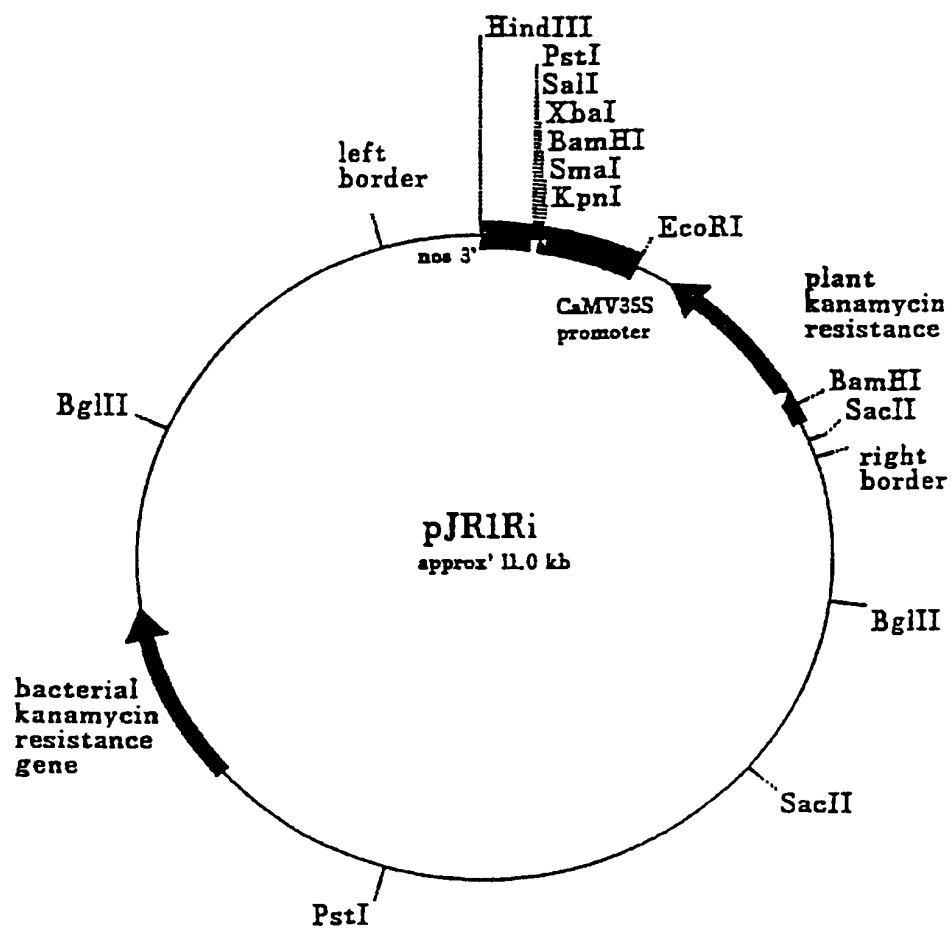


8. A method as claimed in claim 7, in which the modified gene specifies phytoene synthase.
9. A modified chloroplast targeting sequence comprising nucleotides 1 to 417 of  
5 SEQ-ID-NO-1

1/2

FIGURE 2

pJR1Ri

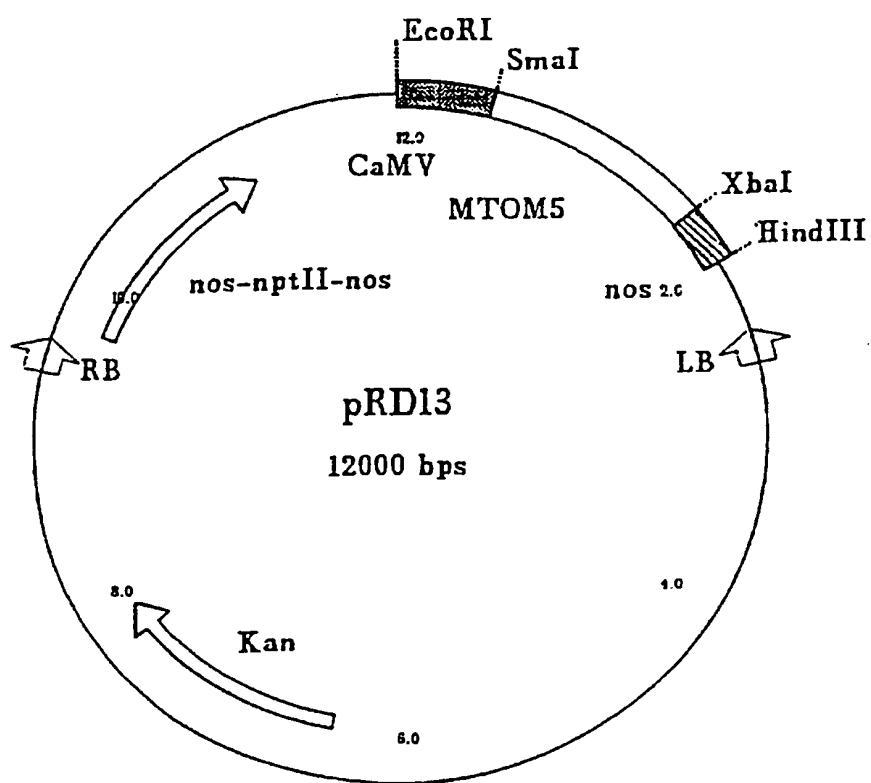


2/2

## FIGURE 3

pRD13

MTOM5 encodes phytoene synthase



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 97/01414

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/67 C12N15/82 C12N15/29 C07K14/415

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FRAY, R.G., ET AL.: "Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway" THE PLANT JOURNAL, vol. 8, no. 5, November 1995, pages 693-701, XP002043131	1-4,6-8
Y	see the whole document	1-4
Y	WO 90 02189 A (UPJOHN CO) 8 March 1990 see the whole document	1-4
X	WO 95 02060 A (ZENECA LTD ;GRIERSON DONALD (GB); FRAY RUPERT GEORGE (GB)) 19 January 1995 see page 18, paragraph 1	1-4

-/--

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

\*A\* document defining the general state of the art which is not considered to be of particular relevance

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\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*Z\* document member of the same patent family

Date of the actual completion of the international search

10 October 1997

Date of mailing of the international search report

17. 10. 97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
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Fax: (+31-70) 340-3016

Authorized officer

Maddox, A

## INTERNATIONAL SEARCH REPORT

Intern 1st Application No

PCT/GB 97/01414

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	BARTLEY, G.E., ET AL.: "A tomato gene expressed during fruit ripening encodes an enzyme of the carotenoid biosynthesis pathway" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, 1992, pages 5036-5039, XP002043132 see the whole document	1-4
X	--- KUMAR, A.M., ET AL.: "Potato plants expressing antisense and sense S-adenosylmethionine decarboxylase (SAMDC) transgenes show altered levels of polyamines and ethylene: antisense plants display abnormal phenotypes." THE PLANT JOURNAL, vol. 9, no. 2, February 1996, pages 147-158, XP002043133 see figure 2	1,2
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X	--- WO 96 02650 A (ZENECA LTD ;KARVOUNI ZOI (GR); JOHN ISAAC (GB); TAYLOR JANE (GB);) 1 February 1996 see the whole document	7,8
X	--- WO 96 13149 A (AMOCO CORP) 9 May 1996 see page 18, line 23 - line 31	7,8
X	--- WO 95 34668 A (BIOSOURCE TECH INC) 21 December 1995 see examples 6,7	7,8
A	--- WO 94 09143 A (MOGEN INT ;TUNEN ADRIANUS JOHANNES VAN (NL); MOL JOSEPHUS NICOLAAS) 28 April 1994 see page 7, line 20 - line 26 see page 10, line 28 - page 11, line 10	1-6
A	--- FINNEGAN, J., ET AL.: "Transgene inactivation: plants fight back" BIOTECHNOLOGY, vol. 12, September 1994, pages 883-888, XP002043134 see the whole document	1-8
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International Application No

PCT/GB 97/01414

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	FLAVELL, R.B.: "Inactivation of gene expression in plants as a consequence of specific gene duplication" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 91, April 1994, WASHINGTON US, pages 3490-3496, XP002043136 see the whole document ---	1-8
A	FRAY R G ET AL: "IDENTIFICATION AND GENETIC ANALYSIS OF NORMAL AND MUTANT PHYTOENE SYNTHASE GENES OF TOMATO BY SEQUENCING, COMPLEMENTATION AND CO-SUPPRESSION" PLANT MOLECULAR BIOLOGY, vol. 22, pages 589-602, XP002017524 see the whole document ---	1-5
A	WO 91 09128 A (ICI PLC) 27 June 1991 see the whole document ---	7,8
A	WO 90 01551 A (ROGERS JOHN C) 22 February 1990 see the whole document -----	1

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PCT/GB 97/01414

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information on patent family members

International Application No

PCT/GB 97/01414

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